

U.S. PATENT APPLICATION

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Invention: ALDOSE REDUCTASE INHIBITOR AND A PROCESS FOR THE
PREPARATION THEREOF

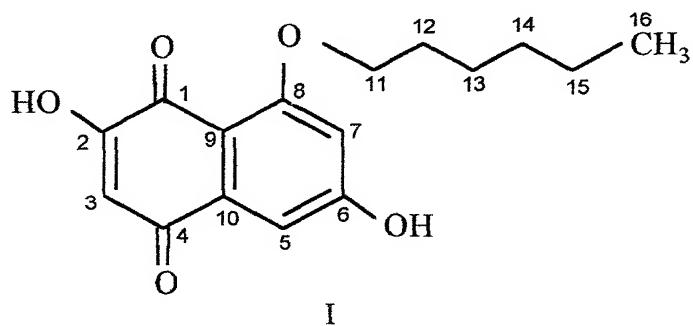
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SPECIFICATION

ALDOSE REDUCTASE INHIBITOR AND PROCESS FOR PREPARATION THEREOF

Field of the invention

The present invention relates to a novel aldose reductase inhibitor. More particularly, the present invention relates to a novel aldose reductase inhibitor of formula I derived from cultures of *Aspergillus niger* CFR 1046 and pharmaceutically acceptable derivatives thereof.



Background of the invention

Diabetes mellitus and its disabling complications which include blindness, renal failure, limb amputation, myocardial infarction affect millions of people (Schumacher, M.P. *Diabetes* 44 (1995)1355-1361). In the absence of or in cases of insufficient insulin, glucose accumulates in the blood of diabetics. Certain cells such as those in the peripheral nerves, the lens of the eye, or parts of the kidneys, however, do not need insulin to get glucose. As a result, maximum damage such as blindness, neuropathy and kidney damage occurs in such sites in diabetics. Nerve damage in diabetes is largely caused by loss of the insulating myelin sheath around nerves, which in turn causes message transmission to be erratic and painful, or non-existent. The reason this occurs is that when glucose enters these cells, it is converted into sorbitol by the enzyme aldose reductase (Alditol:NADP⁺ 1-oxidoreductase, EC 1.1.1.21) which catalyses the NADPH-linked reduction of a broad group of aldoses to respective alduronic acids (Wilson K.W., Boren K.M. and Gabbay K.H., *Science* 257 (1992) 3). Sorbitol is then slowly converted into fructose by sorbitol dehydrogenase. Neither sorbitol nor fructose can cross the membrane and leave the cell easily, thereby causing damage as they accumulate. The resulting changes include changes in osmotic pressures, alterations in redox state of pyridine nucleotides (increased NADH/NAD⁺ ratio) and depleted intracellular levels of myoinositol (Larson ER, Lipinski CA and Sarges R *Med Res Revs* 8 (1988) 159-186).

Since diabetics have larger than normal amounts of glucose in the blood, the damage can lead to the above-mentioned problems. Certain substances present naturally in foods, block aldose reductase and prevent cell damage. Vitamin C, Alpha Lipoic Acid, Licorice, and

various citrus or other antioxidants, e.g. quercetin, are aldose reductase inhibitors (Kim H.Y. and Oh J.H., *Biosci. Biotech. Biochem* 63 (1999) 184-188). Inhibitors against these enzymes thus have a potential application in both the food and medical sector.

Traditionally in Japan, some kampo medicines (traditional oriental herbal prescriptions) have long been used for the treatment of diabetic neuropathy (Aida, K., Tawata, M., Shindo, H., Onaya, T., Sasaki, H., Yamaguchi, T., Chin, M., Mitsuhashi. *Planta Med*, 56 (1990) 254-258). The authors investigated the components of *Glycyrrhizae radix*, a constituent of some kampo medicines, and isolated six compounds (GUs 9-17). Among these, GU-17, identified as isoliquiritigenin, had the most potent aldose reductase inhibiting activity.

Isoliquiritigenin inhibited rat lens aldose reductase with an IC_{50} of $3.2 \times 10(-7)$ M, using DL-glyceraldehyde as a substrate. It inhibited sorbitol accumulation in human red blood cells in vitro, with an IC_{50} of $2.0 \times 10(-6)$ M. Isoliquiritigenin, when administered via an intragastric tube to diabetic rats, suppressed sorbitol accumulation in the red blood cells, the sciatic nerve, and the lens as effectively as ONO-2235. These results suggest that isoliquiritigenin may be effective in preventing diabetic complications.

Among the natural compounds, flavonoid compounds such as quercetin, quercetin, naringinin and hesperidin are reported to be useful and potent aldose reductase inhibitors (Aida, K., Tawata, M., Shindo, H., Onaya, T., Sasaki, H., Yamaguchi, T., Chin, M., Mitsuhashi. *Planta Med*, 56 (1990) 254-258).

The presently known chemically synthesized aldose reductase inhibitors are Tolrestat, a chemically synthesised inhibitor with an IC_{50} of 3.5×10^{-8} M; and Sorbinil (Hollis HD, Johnson JL and Werbel LM. *J Med Chem*. 27 (1984) 255-256).

Some inhibitors obtained through the fermentation route are Thiazocins obtained from the fermented broth of *Actinosynnema* sp (Ozasa T, Yoneda T, Hirasawa M, Suzuki K, Tanaka K, Kadota S and Iwanami M. *J Antibiot* 44 (1991) 768-773), Thermorubrin and 2-hydroxy acetic acid from *Thermoactinomyces* sp UTA 8 (Hayashi K, Dombou M, Sekiya M, Nakajima H *J. of Antibiot.* 48 (1995) 1345 – 1346), Salfredins from *Crucibulum* sps (Matsumoto K, Nagashima K, Kamigauchi T, *J of Antibiot.* 48 (1995) 439-446) and YUA001 from *Corynebacterium* sp (Bahn Y, Park J, Bai D, Takase S, Yu J, *J of Antibiot.* 51 (1998) 902-907) which has an IC_{50} of 1.8 mM against pig kidney aldose reductase.

The search is constantly on for new sources of aldose reductase inhibitors from natural sources that are inexpensive and show better activity.

Objects of the invention

It is an object of the invention to locate and identify new natural sources for aldose reductase inhibitors that are more economical and show better activity.

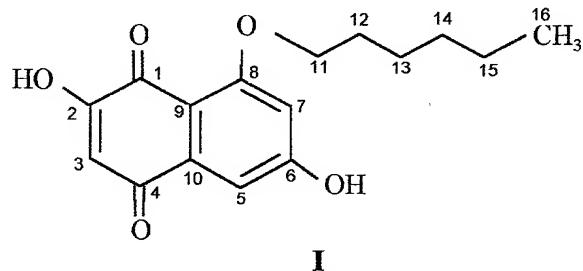
It is another object of the invention to provide new aldose reductase inhibitors that show improved activity and are obtained from natural sources.

A further object of the invention is to provide process for the extraction of aldose reductase inhibitors that are economical and more efficient.

Summary of the invention

These and other objects of the invention are achieved by a novel aldose reductase inhibitor derived through the fermentation of certain microorganisms.

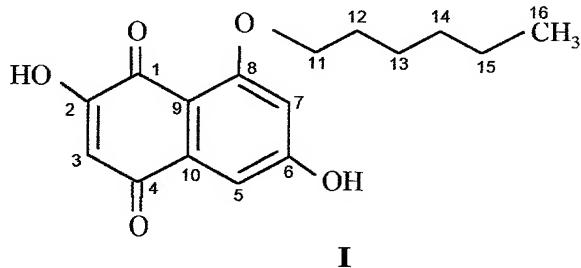
Accordingly, the present invention relates to a novel aldose reductase inhibitor of the formula I below and its pharmaceutically acceptable derivatives



In one embodiment of the invention, the aldose reductase inhibitor is a rat lens aldose reductase inhibitor.

In another embodiment of the invention, the aldose reductase inhibitor of formula I is 2, 6, dihydroxy, 8, hexoxy, 1, 4, naphthaquinone.

The invention also relates to a process for the isolation of a novel aldose reductase inhibitor of the formula I below



the process comprising culturing *Aspergillus niger* CFR 1046 and isolating said aldose reductase inhibitor.

In another embodiment of the invention, the aldose reductase inhibitor isolated from *Aspergillus niger* CFR 1046 is 2, 6, dihydroxy, 8, hexoxy, 1, 4, naphthaquinone.

In one embodiment of the invention, the aldose reductase inhibitory compound of formula I is isolated from *Aspergillus niger* CFR 1046 by fermentation.

In another embodiment of the invention, naphthaquinone is isolated from fermentates of *Aspergillus niger* CFR 1046 by solvent extraction.

In another embodiment of the invention, naphthaquinone is isolated from fermentates of *Aspergillus niger* CFR 1046 by column chromatography.

In another embodiment of the invention, naphthaquinone is isolated from fermentates of *Aspergillus niger* CFR 1046 by crystallization.

In a further embodiment of the invention, the compound of formula I is extracted from *Aspergillus niger* CFR 1046 from fermented potato dextrose broth using ethyl acetate solvent followed by column chromatography and crystallization.

In another embodiment of the invention, the compound of formula I isolated from *Aspergillus niger* CFR 1046 is converted to a pharmaceutically acceptable derivative.

In a further embodiment of the invention, the pharmaceutically acceptable derivative comprises acid or base addition products such as salts of this compound are also useful.

In a further embodiment of the invention, the addition products contain hydrochloride, hydrobromide, sulfate, sodium, potassium, calcium and the like ions.

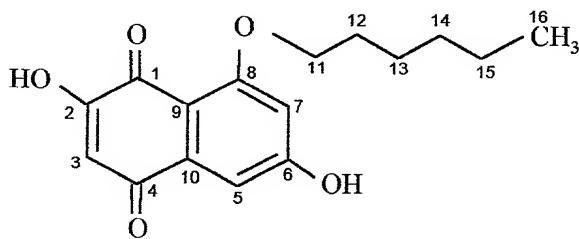
Brief description of the accompanying drawings

Fig. 1 shows multiple quantum coherence transfer spectra (MQCT) of compound I, 2, 6, dihydroxy, 8, hexoxy, 1, 4, naphthaquinone

Fig. 2 shows the mass spectrum of compound I, 2, 6, dihydroxy, 8, hexoxy, 1, 4, naphthaquinone

Detailed description of the invention

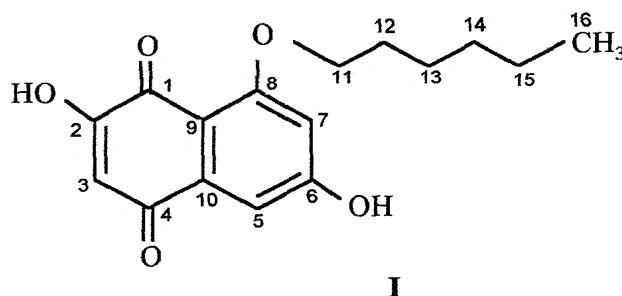
Aspergillus niger CFR 1046 isolated from a sample was found to produce new biologically active substance. This substance was extracted from the fermented potato dextrose broth using ethyl acetate followed by column chromatography and crystallization. The purified compound was identified as 2, 6, dihydroxy, 8, hexoxy, 1, 4, naphthaquinone by spectral analysis.



Thus the fermentation of *Aspergillus niger* CFR 1046 or a mutant thereof and suitable isolation techniques may be used to produce the compound of the investigation.

A biologically pure culture of *Aspergillus niger* CFR 1046, from which the compound of investigation was derived, has been deposited with the American Type Culture Collection (ATCC) in Rockville, MD, and has been added to its permanent collection under its accession code ATCC.

A novel substance was isolated from the fermented broth of *Aspergillus niger* species CFR 1046 originally obtained from the CFTRI Culture Collection. The purified compound was found to conform to structure I



The compound, named by the inventors as SS 140104, is designated as 2, 6, dihydroxy, 8, hexoxy, 1, 4, naphthaquinone. Structure was confirmed by spectral analysis.

Acid or base addition products, e.g., salts, of this compound are also useful. Among the preferred addition products are those containing the ions hydrochloride, hydrobromide, sulfate, sodium, potassium, calcium and the like.

TAXONOMY

Morphology

The fungal mycelium on Czapek's solution agar had abundant erect and crowded conidial structures, which was brownish black in color. Conidial heads were divergent, dense and colonies were wrinkled on the reverse surface. Sclerotia were not observed.

Cultural and physiological characteristics

The growth characteristics of CFR 1046 on malt-extract-agar were similar to that of Czapek's solution agar. But the colonies were dark and less dense. The culture grew well at ambient temperature of 26°C, 30°C. Conidial walls were smooth and thick. The length of Conidiophores was 0.67-1.13 mm. Table 1 shows the growth of CFR 1046 in the presence of various carbon and nitrogen sources.

Table 1: Growth of CFR 1046 on Czapek's agar in the presence of various carbon and nitrogen sources.

C-Source	growth	reverse side color Of the plate	spores
Maltose	very good,	not wrinkled	dark, dense
Glucose	good,	not wrinkled	dark, dense
Fructose	good	not wrinkled	very dark, very dense
Cellobiose	less	not wrinkled	dark, dense
Meso-Inositol	good	not wrinkled	dark, less dense
Rhamnose	good	not wrinkled	dark, less dense
Mannitol	less	not wrinkled	dark, less dense
Xylose	less	not wrinkled	dark, dense
Arabinose	good	not wrinkled	brownish, less dense
N-Source (C-Source : Sucrose)			
Ammonium	good, non -	wrinkled	very dark, dense
Sulfate	sporulating margin		
Ammonium	good	wrinkled	very dark, dense
Nitrate			
Ammonium	good, prominent	wrinkled	very dark, dense
Chloride	non sporulating margin		

Taxonomic position

The strain of *Aspergillus niger* CFR 1046, isolated locally, was taken from the CFTRI Culture Collection. It was further characterized. The characteristics indicated that the strain belongs to *Aspergillus niger* group. According to the descriptions of Raper and Fennell (Raper KB and Fennell DT (1965) The Genus *Aspergillus*; The Williams and Wilkins Co, Baltimore, pp 293-310), the strain CFR 1046 is related to *Aspergillus niger* V. Tiegh.

FERMENTATION**Stock Culture and Flask Fermentation**

Strain CFR 1406 was propagated on Potato Dextrose Agar (Hi Media Mumbai, India) slant composed of soluble starch 0.4 % and glucose 2 %. After incubation for 4 days at 30°C, a portion of the mature agar slant was inoculated into 100 ml of Potato Dextrose in a 500 ml Erlenmeyer flask and incubated at 30°C on a rotary shaker at 250 rpm. The inoculated flasks were incubated for 8 days at 30°C.

Isolation and Purification

Fermented broth (3 L) was treated with ethyl acetate (2 liter) for two hours followed by cheesecloth filtration to remove the biomass and the organic extract was separated from the broth by a separating funnel. This organic extract was dried over anhydrous sodium sulfate and concentrated in vacuo to afford a crude solid (0.42 gm). The residue was re-suspended in chloroform and dry coated on 0.5 gms silica gel. This was loaded on a 5 gm (60-120 mesh) silica gel column (25 X 1 cm;) packed in hexane. The compound was eluted as a deep yellow band in chloroform:ethylacetate (9:1) fraction. Solvent was evaporated in vacuo to obtain 25 mgs of amorphous red powder, designated as Compound 1.

Physico-chemical properties

Compound 1, is an amorphous red powder and is soluble in methanol, ethyl acetate, dimethyl sulphoxide, diethyl ether, sodium bicarbonate solution, sodium carbonate solution and sodium hydroxide solution, slightly soluble in chloroform and hexane, but insoluble in water. The physico-chemical properties are given in Table 2. EI-MS spectra of the compound showed the molecular ions at m/z 205. The fragmentation analysis is also given in Table 2.

Table. 2: Physico-chemical properties of compound 1.

Nature: Red amorphous powder.

Melting Point: decomposes at 170 - 175 °C

λ_{\max} nm (ε) at pH 3.8 : 232 (8,800),
265 (12,700),
310 (5,900).

λ_{\max} nm (ϵ) at pH 9.0 : 226 (21,600),
286 (15,400),
355 (5,800).

IR: 3437, 2995, 2912 and 1660 cm^{-1} .

Molecular formula: C₁₆H₁₈O₅

EI-MS m/z: 290 - 85 = 205 ^{M+1}

$$206 - 28 = 178$$

M⁺ - CO

$$178 - 28 = 150$$

-CO

$$150 - 42 = 108$$

$$108 - 27 = 81$$

HPLC (Rt) : 4.5 min (column: RP-C18, 25 X 4.6mm, Mobile phase: methanol, 0.5ml/min, 265 nm)

GC (Rt): 21.4 min (column: HP-5, Conditions: 50⁰C, 2 min, 10⁰C/min; 220⁰C, 2min, 15⁰C/min; 270⁰C, 20min.)

The molecular formula of compound was established as C₁₆H₁₈O₅, based on the mass spectra and ¹H and ¹³C-NMR spectra (Table 3).

Table 3: ¹H NMR spectra (500 MHz in DMSO-d₆) and ¹³C NMR spectra (500 MHz in DMSO-d₆) of compound 1

	¹ H (δ , ppm)	¹³ C (δ , ppm)
16 CH ₃	0.8	29.9 ^a
11 CH ₂	1.0	31.5
12, 13, 14 CH ₂	1.2	
15 CH ₂	1.5	39.9 – 40.9
H – 7	6.0	111.1
H – 3	6.5	108.9
H – 5	6.95	108.2
4-CO	-	190.83 ^a
1-CO	-	181.7 ^a
6-OH	-	164.4 ^a
2-OH	-	163.6 ^a
8-O	-	160.9 ^a

^a Carbon-13 assignments are interchangeable.

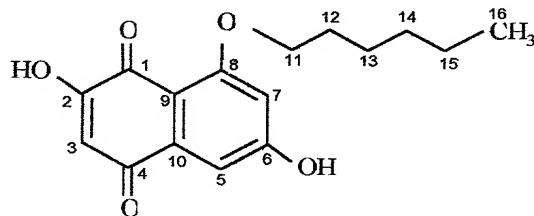
The physico-chemical data are summarized in Table 2. The UV spectra of this substance had an absorption maxima at pH 3.8 are 232, 265, 310 and at pH 9.0 are 226, 286, 355. The IR spectrum (Fig 1) of compound 1 shows typical absorption bands at 3437, 2995, 2912 and 1660 cm⁻¹. The ¹H-NMR spectral data (Fig 2) of this compound and the ¹³C-NMR spectral data (Table 3 and Fig 2) and the Mass fragmentation Spectra (Fig 3) of the compound are given

Structure determination

The spectral characteristics and other properties of the compound isolated are shown in Table 2 and Table 3 above. The compound exhibits UV absorption at 232 nm, 265 nm and 310 nm at pH 3.8. However at pH 9.0, while the λ_{max} at 232 nm showed a hypochromic shift to 226 nm both the 265 nm peak and 310 nm peak showed a bathochromic shift to 286 nm and 355 nm respectively. This indicates that there are ionizable phenolic groups on aromatic

rings. The broad IR absorption at 3437 Cm⁻¹ indicates –OH stretching frequencies. The peak at 2995 Cm⁻¹, 2912 Cm⁻¹ indicate aromatic C-H stretching. A broad ketonic absorption bond in IR spectrum at 1660 Cm⁻¹ indicating probably hydrogen bonded keto group. Another conformation of stretching was obtained from two dimensional heteronuclear multiple quantum coherence transfer spectra (2DHSQC). An aliphatic group corresponding to six carbons were observed at 0.8 (CH₃) and 0.8, 1.0, 1.2 and 1.5 (5 -CH₂) ppm. The –CH₂ at 1.0 ppm might have come from –CH₂ group of hexyl moiety being subjected to ring current effect of the aromatic group present. Three separate single signals were seen at 6.0, 6.5 and 6.95 ppm, indicating aromatic protons. The corresponding ¹³C signals are as follows;

29.9 (–CH₃), 31.5 (–CH₂), 39.9 to 40.9 (–CH₂), 111.1 (Ar-H), 108.9 (Ar-H), 108.2 (Ar-H), 190.8 (CO), 181.7 (CO), 164.4 (C_{Ar}- OH), 163.6 (C_{Ar}- OH), 160.9 (C_{Ar}- O). EIMS analysis showed a parent peak at 205 (M⁺) other fragment peaks like 178 (M⁺-CO), 150 (-CO), 108 and 81 clearly indicating aromatic group of the molecule. However, parent ion peak was distinctly missing. Based on EIMS and 2DHSQC analysis the proposed structure is given below:



Compositions and Methods

In this description of the invention, references to the compound of formula I is intended to include all pharmaceutically acceptable derivatives of the same.

The novel compounds of the invention can be used in a variety of pharmaceutical dosage forms. Thus, oral, parenteral, nasal, topical, buccal, ocular and other forms can be used. When such forms are formulated they will include pharmaceutically acceptable excipients such as colorants, carriers, perfumes, stabilizers, flow modifiers and the like in suitable amounts (i.e., from 0.001 to 0.99wt%).

The compound of the invention is useful in methods of inhibiting the effects of rat lens aldose reductase

The compound of formula I may also be used to treat a host, preferable a mammal, which is suffering from a disorder associated with a metabolism of Aldose reductase, such as

those arising from diabetes related complication such as neuropathy, nephropathy and cataract.

Acid or base addition products, e.g., salts, of this compound are also useful. Among the preferred addition products are those containing the ions hydrochloride, hydrobromide, sulfate, sodium, potassium, calcium and the like.

The following example illustrates the Aldose reductase inhibitor effects of the novel compound.

Experimental

Lens were collected from rat eyes and homogenized with sodium, potassium phosphate buffer (0.135 M, pH 7.0) containing 0.5 mM of phenyl methyl sulphonyl fluoride and 10 mM of β -mercaptoethanol. The homogenate was centrifuged at 10,000 r.p.m for 30 minutes at 4 $^{\circ}$ C. The supernatant was taken as enzyme source. Enzyme reaction was carried out at 25 $^{\circ}$ C in a quartz cuvette with a 1 cm light path. The assay mixture contained 100 μ L of nicotinamide adenine dinucleotide phosphate tetra sodium salt (from a stock solution of 9.6 mM), 100 μ L of DL-glyceraldehyde (from a stock solution of 250 mM), 10 μ L of inhibitor dissolved in dimethyl sulphoxide, sodium-potassium-phosphate buffer (0.135 M, pH 7.0) to make up the total volume to 3 mL and the reaction was initiated by the addition of 200 μ L of enzyme solution. The enzyme reaction was monitored by the decrease in absorbence at 340 nm and compared with enzyme reaction without inhibitor.

Results

The compound (I) was discovered in the fermented bran of a species of *Aspergillus niger* CFR 1046. The compound was successfully purified to homogeneity. The IC₅₀ value of the compound against rat lens aldose reductase inhibitory activity was determined to be 26.2 μ M.

Reasonable variations, such as those that would occur to a skilled artisan, can be made herein without departing from the scope of the invention.